April 2014

Investigating the Role of Multimerization in human Torque Teno Virus VP3 Cancer Cell Specific Apoptosis

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Investigating the Role of Multimerization in human Torque Teno Virus VP3 Cancer Cell Specific Apoptosis

A Major Qualifying Project

Submitted to the Faculty of

WORCESTER POLYTECHNIC INSTITUTE

In Partial Fulfillment of the Requirements for the
Degree of Bachelor of Science

By

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April 30, 2014

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Table of Contents

Abstract

The human Torque Teno Virus Viral Protein 3 (TTV VP3) has been shown to induce apoptosis in cancer cell lines via a p53-independent pathway similar to its homolog Apoptin, demonstrating its potential as a cancer therapeutic. Apoptin forms aggregates in the nucleus while TTV VP3 localizes in the cytoplasm. In order to determine whether TTV VP3 forms multimers like Apoptin and if multimerization is essential for apoptosis induction, GFP and FLAG-tagged VP3 constructs were created and transfected into the lung cancer H1299 cell line and analyzed for TTV VP3 protein interaction by co-immunoprecipitation studies.

Introduction

TTV Background

Although the human Torque Teno Virus (TTV) was discovered in 1997, little is still understood about its function and pathogenicity. Initially discovered in Japanese patients exhibiting hepatitis symptoms who tested positive for known hepatitis viruses, TTV (also known as Transfusion Transmitted Virus) was thought to be responsible for liver disease and cirrhosis. Later tests indicated that different strains of TTV are present in over 90% of the populations in most countries, with a higher prevalence in patients with liver disease [3]. There is no proven correlation between TTV and the onset of liver problems, but transfusion patients and liver patients proved to have a higher viremia than healthy individuals. One study argues that co-infection of TTV and Hepatitis E Virus promotes advancement of hepatic inflammatory responses but that severe liver disease suppresses TTV infection [2]. This unusual behavior of TTV in vivo is not well understood. However, the responsiveness of TTV to damaged cells, and the fact that viral replication (but perhaps no other viral protein function) is hindered, could indicate the virus's sensitivity to apoptotic cellular pathways. The peculiar commensal behavior with human cells, especially in the liver, and Hepatitis E, as well as the sensitivity to damaged-cell pathways, make TTV an interesting subject for cancer therapy research.

TTV Genomics and Classification

In an effort to understand the prevalence and clinical effects of TTV, researchers are focusing on TTV's genetics and molecular behavior. Through a study conducted by

Simmonds, et al, it was determined that TTV is a non-enveloped virus with a single stranded DNA (ssDNA) genome [14]. This and other studies proved the high stability of the virus, showing it could withstand conditions in the bile, Tween 80 treatment, high temperatures, and detergents, likening TTV to parvoviruses [3]. Initial DNA studies suggested the genome was linear, until it was discovered that a GC-rich area on each extremity forms a circular molecule [12]. Because of the similarities to both parvoviruses and circoviruses, the taxonomy of TTV has changed within the last few decades. As of 2006, it is considered a part of the anellovirus family [11], a viral family consisting entirely of the TTVs of different species, including the human Torque Teno Virus [1].

Even within the human Torque Teno Virus (hTTV) classification, there are high levels of genetic variability: there are 38 known genotypes that have been further classified into one of five phylogenetic groups [12]. This is unusual for a virus with only 3.8 kb and unknown pathogenicity. While there is only 60% homology among the genotypes, the three open reading frames (ORFs) are conserved 70-100% within each strain; they code for viral proteins 1, 2, and 3 (VP1-3) respectively. It is believed that VP1 is involved in the construction of the viral capsid. ORF1 contains several conserved replicase protein motifs and is highly basic near its N - terminus, two qualities which Noteborn et al determined to be present in circoviral capsid protein formation. The protein encoded by ORF2, VP2, is less understood, but as it is similar in sequence to VP2 of the chicken anemia virus (CAV) which is classified as a structural scaffold protein, it is likely the TTV VP2 is also involved in a scaffold pathway or structural assembly. VP3, the focus of this particular study, is coded by ORF3 and is considerably smaller than VP1 and VP2.

Comparison to the Chicken Anemia Virus

These ORFs and resulting viral proteins are very similar to those of the Chicken Anemia Virus (CAV), a circovirus that is known for its viral protein 3 (VP3), nicknamed Apoptin. Apoptin has shown to induce apoptosis in cancer cells independent of a p53 pathway while leaving primary cells intact [7]. P53 is a tumor suppressor that initiates a DNA repair pathway for damaged cells by arresting the cell cycle between the G1 and S phases. This protein is often the target of cancer therapies as it has the ability to induce apoptosis in cells that are severely damaged. Cancer cells, however, often have dysfunctional p53, if the protein is present at all, making many of these p53-targeted cancer therapies obsolete. Apoptin is able to induce apoptosis regardless of p53 presence in cancer cells, making it highly interesting for broad-spectrum cancer research.

Apoptin localizes in the nucleus of cancer cells and then forms multimers, a function that somehow induces the apoptosis of the cancer cell. Previous studies have suggested the nuclear localization sequence (NLS) and nuclear export sequence (NES) are directly involved in inducing cellular apoptosis, and that the shuttling of Apoptin into the nucleus stimulates the apoptotic induction pathway [7]. However, other Apoptin homologs, such as Porcine Circovirus 1 VP3 (PCV VP3) and TTV VP3, localize in the cytoplasm [4], suggesting the NLS/NES signaling may not be as critical in the apoptosis-inducing process.

TTV VP3, containing 105 amino acids in the TA278 TTV strain, demonstrates significant functional similarity to Apoptin. TTV VP3 has also been referred to as TAIP, or TTV-derived apoptosis inducing protein [8], since it also induces apoptosis in cancer

cells via a non-p53-dependent pathway, although to a lesser extent than Apoptin [7]. While TTV VP3 localizes in the cytoplasm of infected cells, like PCV VP3, Apoptin localizes in the nucleus. However, the apoptotic effect of both Apoptin and TTV VP3 and their protein structure similarities suggests that they might induce apoptosis in cancer cells through similar cellular pathways. Both Apoptin and TTV VP3 induce onco-specific apoptosis, but they localize in different areas. Determining whether these two proteins have any functional similarities earlier on in the induction pathway could lead to cancer therapeutic targets in the future.

Multimerization

Heilman, et.al. determined that Apoptin multimerizes varyingly in different cancer cell lines and suggested that these multimer aggregates contribute to Apoptin's ability to induce apoptosis and localize in the nucleus [7]. Overlapping Apoptin's NES (amino acids 33-46) is a multimerization domain that allows Apoptin to form multimers of about 30 units in the H1299 cell line [9]. Leliveld, et.al. suggested that the Apoptin monomer forms beta-turns with alternating hydrophobic and hydrophilic residues, a motif that would provide ample opportunity for intermolecular interactions. This simple secondary structure remarkably allows for the multimerization complex to form, which can then interact with intracellular signals and perhaps induce apoptosis [9].

Previous TTV VP3 studies, particularly the initial WPI project conducted by Evan-Browning and Orme-Johnson in 2009, observed that TTV VP3 tagged with GFP also formed bright aggregates of varying sizes in the cytoplasm of H1299 cells [6]. These aggregates were oriented close to the nucleus but did not diffuse past the nuclear

membrane. Their size and shape were described as both asymmetric and circular, and both diminutive and largely obtrusive. As suggested by Evan-Browning and Orme-Johnson, this seemingly sporadic aggregate formation could either be the result of random weak protein-protein interactions, driven solely by the high concentration of expressed TTV VP3, or the result of a particular secondary structure that creates a multimerization domain.

Given the behavioral similarities, but the localization differences, of Apoptin and TTV VP3, determining whether TTV VP3 also multimerizes like Apoptin does could suggest that the multimerization process is essential for inducing apoptosis. Discovering additional similarities between these two proteins could also further the evolutionary understanding of these two viruses (and other circoviruses) and their potential function as cancer therapeutics. This project will explore TTV VP3's multimerization capacity and how it relates to Apoptin's multimerization process in an effort to understand the apoptotic pathway that both proteins induce.

Materials and Methods

pEGFP+ TTV VP3 Vector Amplification

pEGFP vector was used to transform NEB 10-beta competent cells (C3019I). 5µL of the vector sample was added to 100µL of the high-efficiency competent cells and allowed to incubate on ice for 10 minutes. The mixture was then heat shocked for 20 seconds at 42°C and placed back on ice for 3 minutes. 900uL of LB broth containing 1X kanamycin was added and the tube was incubated at 37°C on a rotator for 45 minutes. 200µL of the transformation was then plated on a kanamycin agar plate and allowed to incubate overnight at 37°C. Using a sterile pipet tip, one of the resulting colonies was picked and rescreened on a second kanamycin agar plate and allowed to incubate at 37°C, upside down, overnight in order to separate positive colonies from satellite colonies. The following day, one large colony was inoculated into 250mL of LB broth containing 1X kanamycin and incubated at 37°C overnight on the shaker. The Promega Midi-Prep Kit was then used to extract the pEGFP TTV VP3 plasmid. In order to discern whether the resulting extraction contained the desired plasmid, a double digest was conducted on 3µL of the sample using BamH1 and EcoR1. After an hour of incubation at 37°C, the digests were resolved on a 0.9% agarose gel at 90V and post-stained with ethidium bromide to visualize the bands.

3X FLAG Plasmid Construction

Vector Components

A Promega pGEM-T construct containing the TTV VP3 gene of interest was double digested with 1 µL of BamH1 and EcoR1 each. A 3X FLAG vector containing a porcine

circovirus gene was also double digested with 1 µL of BamH1 and EcoR1. Both digests were allowed to incubate at 37°C for an hour and were then resolved on a 0.9% agarose gel at 90V. Post-staining with ethidium bromide was done in order to visualize the bands on the gel. The 3X FLAG vector bands and the TTV VP3 insert bands were excised from the gel and purified using Promega's PCR Clean-up Kit.

Transformation & Colonization

Ligations of the TTV VP3 insert and the 3X FLAG vector were performed in quadruplicate using T4 DNA Ligase and DNA Ligase Buffer, with the insert in excess (6µL) and the vector in limiting (1µL) quantity. The ligations were stored overnight at 4°C. Each ligation reaction was transferred into 100µL of Pro-5 alpha JM109 competent cells and incubated on ice for 15 minutes in order to form DNA complexes. The cells underwent heat shock for 45 seconds before being put on ice for 3 minutes. One negative control of JM109 cells was made without any DNA transformation and two other negative controls of JM109 cells were made using junk DNA from previously failed ligation reactions. 900µL of LB broth was added to each transformed and negative control tubes, which were then incubated at 37°C on a rotator for 45 minutes. 200µL of each competent cell mixture was spread on separate ampicillin agar plates and allowed to incubate upside down for 24 hours. Large colonies were inoculated with a sterile pipet tip into 3mL of LB broth containing ampicillin. Two colonies were taken from each plate, resulting in 8 inoculated cultures. The culture tubes were put on the shaker at 37°C for 24 hours to encourage culture growth.

3X FLAG+TTV VP3 Extraction and Amplification

A total of 8 plasmid DNA extraction procedures were done using Promega's Mini-Prep Kit. Each extraction produced 30μL of purified plasmid DNA extract. To determine whether this purified plasmid was our 3X FLAG construct containing the TTV VP3 gene of interest, a double restriction digest was done on 3μL of each sample using 1 μL of BamH1 and EcoR1 each. After an hour of incubation, the digested samples were resolved on a 0.9% agarose gel at 90V and post-stained with ethidium bromide to visualize the bands.

Each of the 8 samples showed positive results, but 3 samples with the best bands were chosen to amplify our construct. Using the dregs of the cultures used for the Mini-Prep, the competent cells containing our desired 3X FLAG construct were inoculated into 100mL of LB broth containing ampicillin in a large Erlenmeyer flask and put on the shaker at 37°C for 24 hours. Each culture showed sufficient growth and the construct was extracted using Promega's Midi-Prep Kit following the centrifugation protocol. These extractions resulted in three 600μL samples of plasmid DNA. To discern that these extractions contained our desired 3X FLAG and TTV VP3 construct, a double digest was performed on 5μL of each sample using 1 μL of BamH1 and EcoR1 each. After an hour of incubating at 37°C, the digests were resolved on a 0.9% agarose gel at 90V and post-stained with ethidium bromide to visualize the bands. Once bands were visualized, the samples were sent to the lab to be sequenced.

Maintaining H1299 Cell Culture

H1299 lung cancer stock cells were suspended in D10 media (DEMEM-HEPES, fetal bovine serum, glutamine, gentamicin) in growth-enhanced T-25 tissue culture flasks and kept at 37ºC for optimal growth. Once the cells had adhered to the flask and had reached 80% confluence, the D10 media was aspirated and the cells were washed with 2 mL of PBS. Trypsin (1mL) was added to the flask and curtained over the cells 5-10 times before being quickly aspirated. After approximately four minutes, the cells were visualized under the microscope to insure they were no longer adhered to the flask and were then resuspended in 5mL of warm D10 by gently pipetting the media up and down. An aliquot of the resuspended cells (0.5-1mL) was then transferred to a new T-25 flask and 5-6mL of fresh D10 was added. The new flask containing the resuspended cells was then placed in the 37ºC incubator to optimize the H1299 cell growth, and the passage process was repeated every 2-3 days when cells reached 80% confluence.

Wild Type TTV VP3 Transfection & Harvest

In order for the wt TTV VP3 fusion proteins to be analyzed in cancer cell conditions, the 3X FLAG and GFP TTV VP3 constructs were transfected into the H1299 cells. Following Qiagen's transfection protocol for 100mm plates, cells were passed one day prior to transfection and were transfected about 24 hours later when they had reached 40-60% confluence. In order to optimize the transfection efficiency, 4µg of each TTV VP3 construct was used instead of the recommended 2µg DNA total. 24-48 hours post-transfection the cells were visualized with the fluoroscope to verify the expression of the GFP TTV VP3 fusion protein. The cells were then harvested by aspirating the D10, washing in PBS, scraping the cells, and resuspending them in 200µL of PBS. The cell

suspension was added to an Eppendorf tube and spun down (1500g for 3 minutes) to create a cell pellet. The PBS supernatant was discarded, then 100µL of lysis Buffer X (pH 8.5) was pipetted gently to break apart the pellet and lyse the cells and was left to incubate for 15 minutes. The cell extract sample was then clarified by centrifugation (1500g for 5 minutes) to precipitate insoluble lipids and other heavy cell debris. The clarified extract (supernatant) was carefully transferred to a fresh tube to be used for immunoprecipitation.

Truncated TTV VP3 Transfection & Harvest

Following Qiagen's Effectene protocol for 6 well plates, H1299 cells were transfected with the DNA constructs listed in Table 1 (See Tables Section). The cells were harvested 48 hours post-transfection by collecting the media into a 15mL conical tube, scraping and collecting the cells, and washing the remaining cells in PBS and again adding the suspension to the conical tube. The six tubes were subjected to centrifugation (1500g for 5 minutes) to collect the cell pellets, which were then resuspended in 100µL lysis buffer X (pH 7.4 + PI) and clarified. The supernatants were transferred to clean Eppendorf tubes to be used for immunoprecipitation.

TTVVP3 Controls: Transfection, Harvest and Fluorimetry

The above process was repeated using the DNA constructs listed in Table 2 (See Tables Section). After lysing the cells with lysis Buffer X (pH 7.4), the fluorescence of each sample was read by a fluorescence spectrophotometer scanning from 470-600nm and observing behavior at 510nm (the emission peak of GFP) in order to track the presence of

GFP fusion proteins in the samples. The fluorescence of the samples was gathered again after clarifying the extract and again after immunoprecipitating with the EZview beads.

FLAG Immunoprecipitation and Western Blotting

EZview Red ANTI-FLAG M2 Affinity beads were added to the clarified cell extracts (10µL were added to extracts from 6 well-plated cells, 15µL were added to extracts from 100mm-plated cells) and were placed on a rotator at 4°C for four hours. In order to elute any bound protein from the affinity beads, the samples were boiled in SDS sample loading buffer at 95°C for 5 minutes before being loaded onto a 16% polyacrylamide gel alongside a pre-stained 2-log protein ladder. The gel was run at 50V until the samples had passed through the stacking gel and then at 70V until the bromophenol blue had reached the bottom.

A transfer sandwich was then assembled using the completed gel and a nitrocellulose membrane that had been soaking in transfer buffer. The sandwich was loaded into the transfer apparatus and run at a constant 200mA for 1 hour. After disassembling the sandwich, the nitrocellulose membrane was put into blocking buffer (TBS-T + 5% powdered milk) for 1 hour. The membrane was then washed 5 X 5 minutes with TBS-T and then blotted for 1 hour with either a 1:5000 monoclonal mouse anti-GFP (Sigma G1546) or 1:5000 mouse anti-FLAG (Sigma F3165) primary antibody diluted in 10mL TBS-T. The membrane was washed again 5 X 5 minutes with TBS-T and then blotted for 1 hour with a 1:5000 polyclonal goat anti-mouse alkaline phosphatase-conjugated secondary antibody (Thermo Scientific Prod #31328) dilution in 10mL TBS-T. The blot was again washed 5 X 5 minutes in TBS-T and then 2 X 5 minutes with TBS before staining with Sigma Fast BCIP/NBT (Sigma B5655) to visualize any bands.

Results

Apoptin has been shown to form multimers in cancer cells, therefore determining whether TTV VP3, an Apoptin homolog, also multimerizes in cancer cells would elucidate whether this multimerization capacity is a necessary function for these proteins to induce onco-specific apoptosis. In order for TTV VP3 multimerization to be investigated using a combination of immunoprecipitation and Western blotting assays, fusion protein plasmids were constructed. A pEGFP construct containing the wild type TTV VP3 gene was verified by the double restriction profile, which proved the presence of a 4000bp pEGFP vector and a 325bp insert when compared against a 2-log DNA ladder (Figure 2, lane 9). Given its usefulness in affinity and immunoprecipitation assays, and the availability of EZ View anti-FLAG beads, it was decided that a FLAG fusion protein would also be constructed by inserting a wild type TTV VP3 sequence into a 3X FLAG CMV-Myc 26 vector. Transforming 3X FLAG and TTV VP3 ligations into JM 109 *e. Coli* bacteria proved successful, as seen in the BamH1 and EcoR1 restriction profile that shows the 6300bp 3X FLAG vector band and the 325bp wt TTV VP3 insert band (Figure 1B). Subsequent sequence analysis further verified the presence of the 3X-FLAG wt TTV VP3 construct with the correct reading frame orientation in each of the three midi-prep extractions (Figure 3), confirming it could be properly expressed in cells.

In order to determine wt TTV VP3's multimerization capacity in human cancer cells in vitro, the pEGFP and 3X-FLAG TTV VP3 constructs were co-transfected into human lung cancer H1299 cells. Co-transfection with 2μg of each construct yielded only 10-15% GFP fusion protein expression when visualized on a fluoroscope. Increasing the

DNA volume to 4μg of each construct yielded over 80% GFP fusion protein expression and therefore successful transfection and GFP TTV VP3 expression. Assuming that TTV VP3 multimerizes, co-transfection and therefore co-expression of the fusion proteins should yield multimers containing both FLAG and GFP tags. Assuming TTV VP3 does not multimerize, both fusion proteins would be expressed but later separated by FLAG-specific immunoprecipitation (Figure 4).

Initial co-immunoprecipitation analysis with a monoclonal M2 goat anti-mouse secondary antibody (KPL-051806) yielded only faint bands at 100kDa and 50kDa (Figure 5). Given the high concentration of the secondary antibody (1500x) that was needed in order to produce this background signal, a new secondary antibody (polyclonal goat anti-mouse alkaline phosphatase conjugated- Thermo Scientific #31328) was used for the subsequent Western blots.

When the wild type-transfected IP samples were again blotted for GFP and FLAG, broad bands at 24kDa, along with the 100kDa and 50kDa bands, were visualized, (Figure 6). Wild type TTV VP3 is 11.6kDa (38.6kDa when fused with GFP and 14kDa when fused with 3X FLAG), so these 24, 50, and 100kDa bands are representative of the mouse antibody and heavy and light chains from the EZ View beads. Although the appearance of this 24kDa band could have been indicating the presence of a GFP-fused protein, it was inconclusive to say given the absence of a negative control.

In order to determine whether this broad 24kDa band was purely representative of the mouse IgG light chain, a TTV VP3 truncation study was conducted. Previous projects had produced four TTV VP3 truncations (aa 1-78, aa 79-105, aa 1-60, aa 61-105) inserted

into a pEGFP vector. H1299 cells were plated into a 6-well dish and co-transfected according to Table 1 (see Tables section). After extracting and immunoprecipitating, the samples were run on two 16% SDS-PAGE gels simultaneously and then transferred to a nitrocellulose membrane. One membrane was western blotted for anti-GFP and the other for anti-FLAG, but other than the differing primary antibodies, the blots were equally treated. The anti-GFP blot showed three bands for every sample at 100kDa, 50kDa, and 24kDa, increasing in intensity. The consistency of these bands, and the fact that the negative control (GFP-wt TTV VP3 only) had a strong signal at 24kDa suggested that this broad band was in fact just background information coming from the EZ View Beads mouse antibodies and was not indicative of GFP's presence in the IP samples.

The Western blot results also did not indicate that TTV VP3 does *not* multimerize as there was no distinct band on the anti-FLAG blot at 14kDa (the molecular weight of TTV VP3 fused with the 3X FLAG peptide) for any of the samples containing FLAG wt TTV VP3. There were indistinct smears or band broadening between 30 and 15kDa, but given the lack of a positive input control signal these indistinct bands are still inconclusive until 3X FLAG TTV VP3 expression and presence in the IP sample can be verified.

In order to test the functionality of the Western blot procedure and track the presence of each TTV VP3 fusion protein, the contents of the 3X FLAG TTV VP3 construct were confirmed by running another BamH1 and EcoR1 restriction profile and four more H1299 cultures were transfected according to Table 2 (see Tables section). In order to ascertain the presence of the GFP fusion protein at each step, the samples were scanned for fluorescence and GFP emission (510nm) directly after cell lysis, after

clarifying the extracts, and after immunoprecipitation. Figure 7 shows the fluorescence results of the 3XFLAG-wt TTV VP3 and GFP-aa1-60 TTV VP3 co-transfected sample. The clarifying extract shows a slight decrease in emission at 510nm from the lysis extract, but, more significantly, the emission peak at 510nm entirely disappears after the FLAG IP and follows the same baseline as the 3X FLAG wt TTV VP3 fluorescence negative control.

Resolving these four IPs alongside an Apoptin control (3X FLAG wt and GFP wt post-FLAG IP) and blotting for both FLAG and GFP indicated the presence of a 17kDa protein in the Apoptin sample on the anti-FLAG blot, but only the usual heavy and light chain bands for the other samples on both blots (Figure 8).

Discussion

Much remains to be understood about the human Torque Teno Virus. Its pathogenicity, if it exists, is still unclear. Originally called the Transfusion Transmitted Virus [2], it was believed that TTV somehow caused liver cirrhosis via blood transfusions, but given its presence in about 90% of the human population it appears to exist complacently in vivo [3]. That being said, the virus easily exists and infects human primary cells with seemingly no adverse effects, making it a potentially powerful vector for gene therapy, drug delivery, or cancer cell targets.

The protein coded by TTV's ORF3 has proved to induce apoptosis in cancer cells similar to its CAV VP3 homolog Apoptin. This apoptotic affect can be visualized directly under the microscope; even within 24 hours many of the cultured cancer cells are obliterated by TTV VP3 and by Apoptin. The mechanism by which this onco-specific cell death is induced is likely similar for both of these proteins given their taxonomical homology and consistent cancer-targeting behavior.

Earlier studies suggested that Apoptin's nuclear localization was critical for arresting the cell cycle [7], but some of its homologs (TTV VP3 included) localize primarily in the cytoplasm and still manage to induce cell death. For this reason the concept of protein multimerization was considered to be another element contributing to the cell-death signaling pathway. Apoptin was found to exist as 30-40 multi-protein complexes by Leliveld et al, and former WPI project studies observed TTV VP3 aggregating similarly through GFP fluorescence studies [8, 6]. The confirmation of Apoptin's multimerization ability and the determination of its multimerization domain

provide us with ample reason to explore whether its hTTV homolog does the same. This study sought merely to determine whether TTV VP3 multimerizes at all in vitro H1299 using similar co-immunoprecipitation assays as Heilman et. al. [8]. Unfortunately, however, this particular study could not conclusively say whether TTV VP3 multimerizes or not.

Immunoblotting failed to both prove and disprove the presence of TTV VP3 multimers in infected H1299 cells. Theoretically, immunoprecipitation with FLAG EZ View beads would pull any FLAG-tagged TTV VP3 out of the clarified H1299 extract. If TTV VP3 multimerizes, most if not all multimers complexes would contain some ratio of GFP and 3X FLAG-tagged protein, so precipitating FLAG-tagged TTV VP3 would pull GFP-tagged TTV VP3 out of solution as well, and blotting for GFP should produce a signal (Figure 4). Assuming TTV VP3 does not multimerize or only multimerized with the FLAG-tagged proteins, western blotting with an anti-FLAG primary should produce a signal. However, blotting for both anti-GFP and anti-FLAG did not produce any evidence towards either of these theories. The lack of signal on the anti-GFP blot (Figure 8A) suggests GFP TTV VP3 was discarded in solution during the IP step, but the lack of a 14kDa band (or a higher molecular weight band if only FLAG-tagged TTV VP3s were multimerizing, excluding the GFP fusion protein) in Figure 8B suggest that neither of the TTV VP3 fusion proteins were precipitated out of solution or able to run through the 16% SDS-PAGE gel.

Having observed significant fluorescence under the fluoroscope, it is known that the GFP-tagged TTV VP3 is definitely expressed in the H1299 cells and begins to induce apoptosis before being harvested. It is possible, however, that the 3X FLAG TTV VP3

could not be properly expressed in the H1299 cells, although this is would be unusual given the results of the sequence analysis (Figure 3), which showed proper insertion and alignment of TTV VP3 within the 3X FLAG vector, and, considering that it had been freshly constructed, it is unlikely that it had been subjected to degrading nuclease activity.

Evan-Browning and Orme-Johnson did observe GFP TTV VP3 aggregates with the fluoroscope [6], and given the particular affinity of FLAG EZ View beads to 3X FLAG fusion proteins, it is also possible that multimer complexes exist and that something about its structure prevents it from being analyzed with this particular protocol. For example, Apoptin's multi-protein complex has been shown to fall out of solution with the insolubles during the clarifying step post-cell lysis (unpublished observation). It is possible that TTV VP3 complexes also fall out of solution with insoluble proteins, but given the intensity of the GFP emission after the clarifying step, we know that GFP TTV VP3 is kept in solution. If the GFP TTV VP3 is indeed multimerizing, it is not falling out of solution before the immunoprecipitation step, but it is still possible that multimers containing the 3X FLAG TTV VP3 are insoluble.

If multi-protein complexes are small enough or soluble enough to stay in solution and they are still not being detected, it is possible that something is preventing the EZ View beads' anti-FLAG from binding to the 3X FLAG epitope. Perhaps the structure of the multi-protein complex is arranged in such a way that the cumbersome 27kDa GFP tag is blocking access to the 3X FLAG tag. It is also possible that the 3X FLAG construct is simply not being expressed. Figure 8 shows that both 3X FLAG TTV VP3 and GFP TTV VP3 were not in the IP sample, and the Western blotting was proven to be functional

given the presence of the 3X FLAG Apoptin band at 17kDa on the FLAG blot. Given the fluorescence observations from the fluoroscope and the fluorimeter, the GFP TTV VP3 is definitely expressed and harvested and is most likely lost after the IP. This information combined with the lack of a 14kDa 3X FLAG TTV VP3 band on the FLAG blots suggests that the 3X FLAG TTV VP3 was either discarded in solution after the IP or was never expressed in the first place.

Future Experiments

Given the inconclusiveness of this project, it is highly recommended that the protocol be optimized and repeated in order to conclusively determine whether TTV VP3 multimerizes or not. Verifying the expression of 3X FLAG TTV VP3 in the H1299 cells, the affinity of the FLAG EZ View beads to the 3X FLAG tag, and the presence of the 3X FLAG fusion protein after the clarifying step would presumably elucidate whether this methodology is functional and whether these results are conclusive.

If subsequent experiments determine that TTV VP3 multimerizes in vitro, the use of the available truncations would help ascertain the location of multimerization domain(s). Point-mutating within these domains to knock out the multimerization capacity could create proteins that exist primarily as monomers. Knocking out the multimerization domain in this way and testing for apoptosis induction in transformed cells could then suggest whether multimerization is necessary for the cell-death pathway to be activated by TTV VP3 and, by extension, Apoptin. Understanding the mechanism by which TTV VP3 interacts with itself and whether this interaction is necessary for onco-specific apoptosis to be induced would bring us one step closer to understanding and harnessing the power of these onco-apoptotic proteins for cancer therapeutics.

Tables

Table 1: Co-transfected H1299 sample descriptions and observations noted 48 hours post-transfection (where * indicates average, ** indicates above average, and *** indicates significant observations).

Co-transfection	Observed Transfection Efficiency	Observed Apoptosis	Observed Localization
3XFLAG-wt TTVVP3 GFP-wt TTVVP3	**	very high	cytoplasm
GFP-wt TTVVP3 (Negative control)	*	low	cytoplasm
3XFLAG-wt TTVVP3 GFP- TTVVP3 aa 1-78	low	low	cytoplasm
3XFLAG-wt TTVVP3 GFP- TTVVP3 aa 79-105	***	low	whole-cell diffusion
3XFLAG-wt TTVVP3 GFP-TTVVP3 aa 61-105	***	***	whole-cell diffusion
3XFLAG-wt TTVVP3 GFP-TTVVP3 aa 1-60	***	***	whole-cell diffusion

Table 2: DNA constructs used to transfect H1299 control samples and apoptosis observations 24 hours post-transfection

DNA constructs used	Observed Apoptosis
3XFLAG-wt TTVVP3	none
GFP-wt TTVVP3	low
3XFLAG-wt TTVVP3 GFP- TTVVP3 aa 1-60	high
3XFLAG-wt TTVVP3 GFP- wt TTVVP3	low

Figures

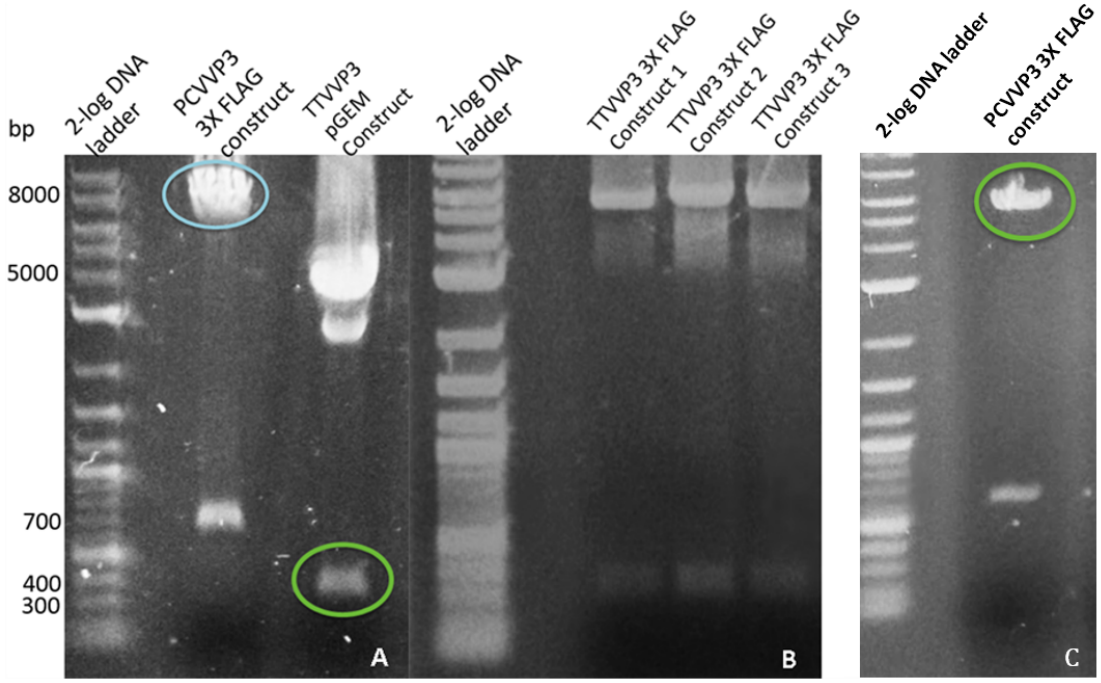

Figure 1: (A)-BamH1 and EcoR1 restriction profile of a 3X FLAG PCVVP3 construct and a pGEM-T wt TTVVP3 construct. The green circle shows the band of the wt TTV VP3 gene that was collected and used as the insert for the 3X FLAG TTVVP3 construct. The blue circle shows the 3X FLAG vector band that was needed but not collected due to clumping. (B)- BamH1 and EcoR1 restriction profile of the new 3X FLAG wt TTV VP3 construct demonstrating proper inclusion of both the 3X FLAG vector (8000bp) and wt TTV VP3 gene (325bp). (C)- Repeat of Figure 1A with a smaller starting volume of the 3X FLAG PCVVP3 construct. The vector band (green circle) was collected and used to create the 3X FLAG TTVVP3 construct seen in Figure 1B.

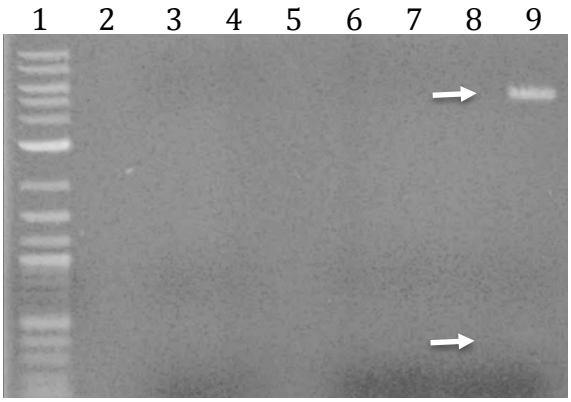

Figure 2: BamH1 and EcoR1 restriction profile of the pEGFP TTV VP3 construct post-amplification (Lane 9) with the vector band at 4000bp and the TTV VP3 insert band at about 325bp.

Figure 3: Sequence analysis of the 3X FLAG TTV VP3 construct verifying proper insertion of the wt TTV VP3 gene with correct reading frame orientation with the 3X FLAG sequence.

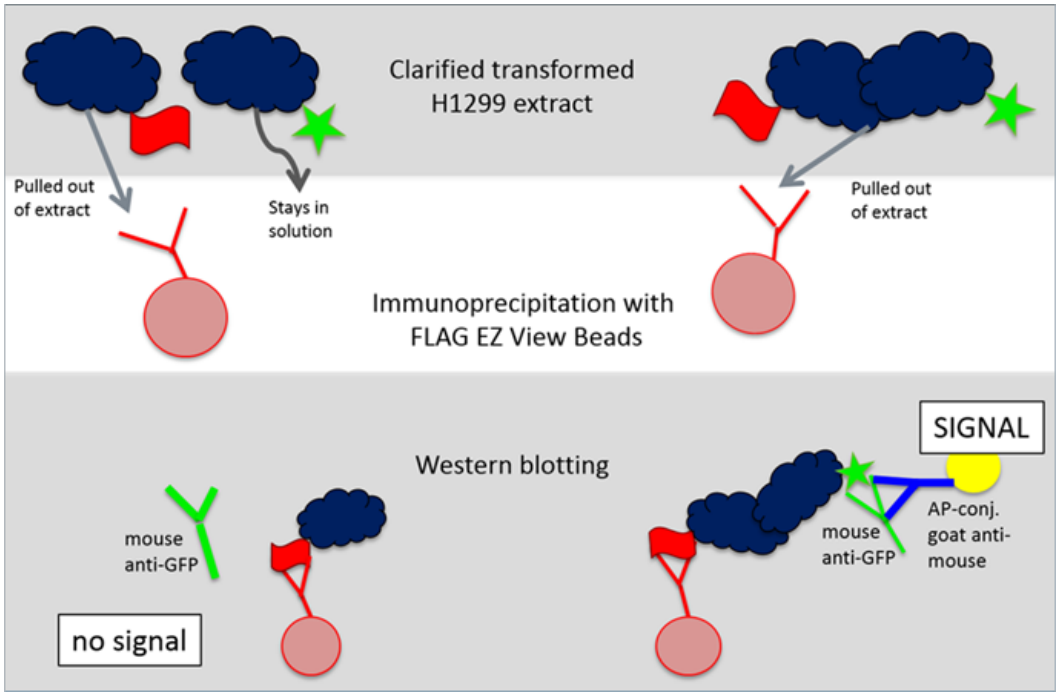

Figure 4: Schematic of the multimerization and co-immunoprecipitation assay hypothesis.

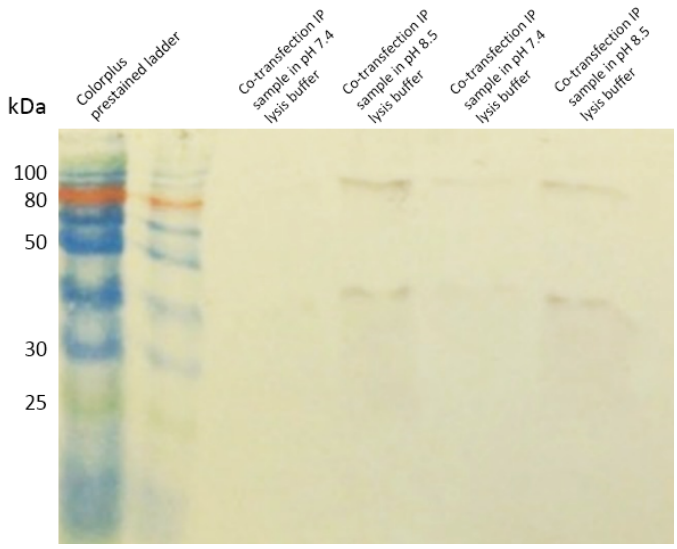

Figure 5: Western blot showing placement of background bands (~100kDa and ~50kDa) when IP samples were blotted for GFP. This blot confirmed a functional primary antibody and that lysis Buffer X produced less background at a pH of 7.4

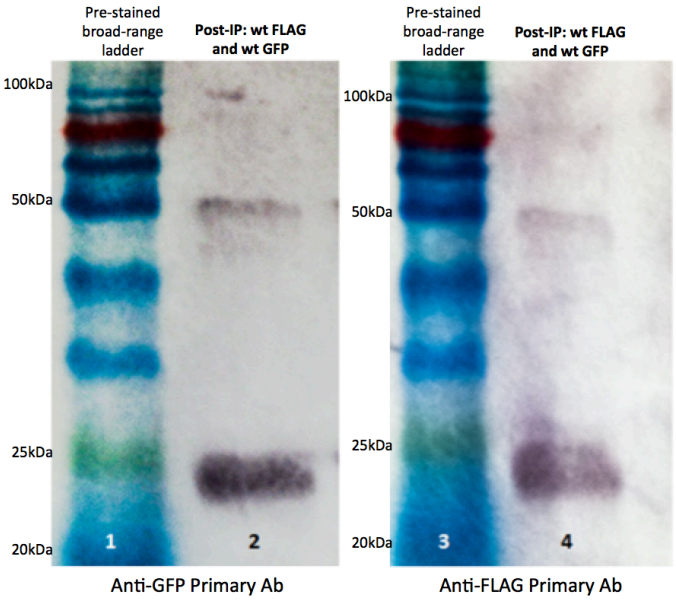

Figure 6: Western blot of an immunoprecipitated co-transfected cell extract blotted for GFP (left) and FLAG (right).

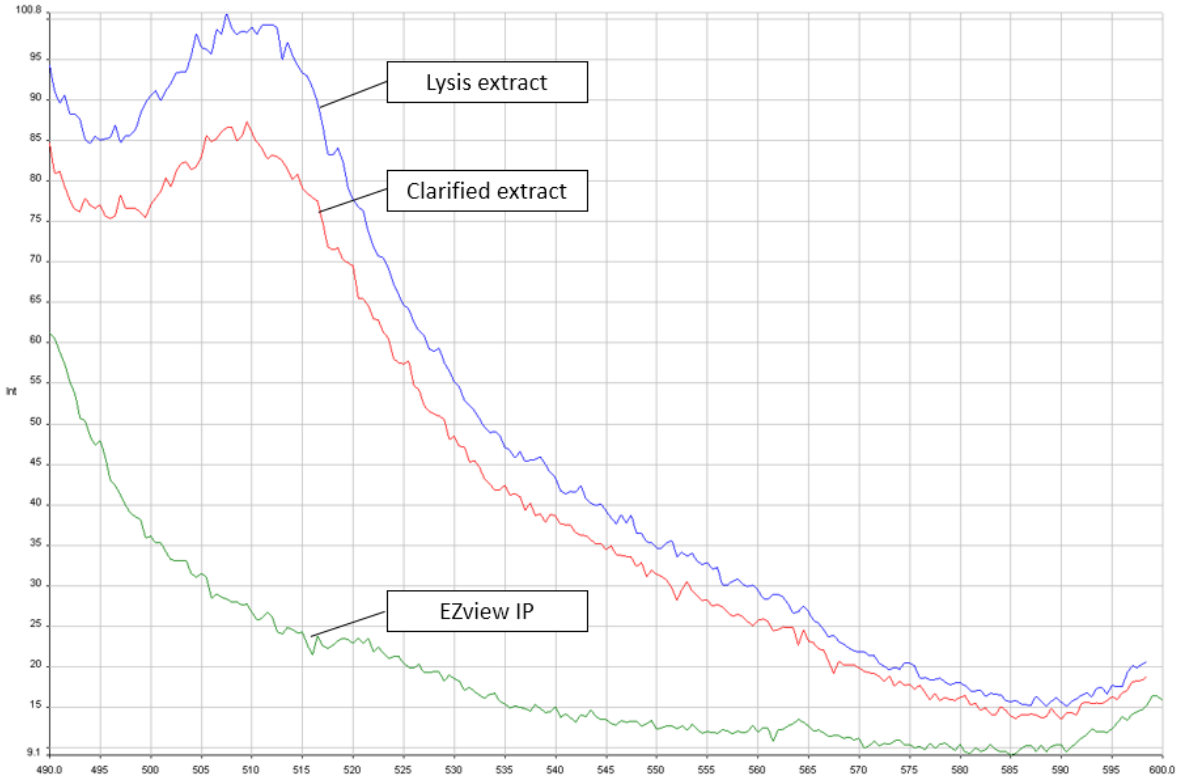

Figure 7: Fluorescence spectra for the 3X FLAG wt TTV VP3 and GFP aa 1-60 TTV VP3 sample. Scanned from 470nm to 600nm, the peaks at 510nm indicate the presence of GFP in both the lysis and clarified extracts. The spectrum after the IP does not show any GFP emission, suggesting that GFP is no longer in the sample or that it is quenched by being tightly packed on the EZ View beads.

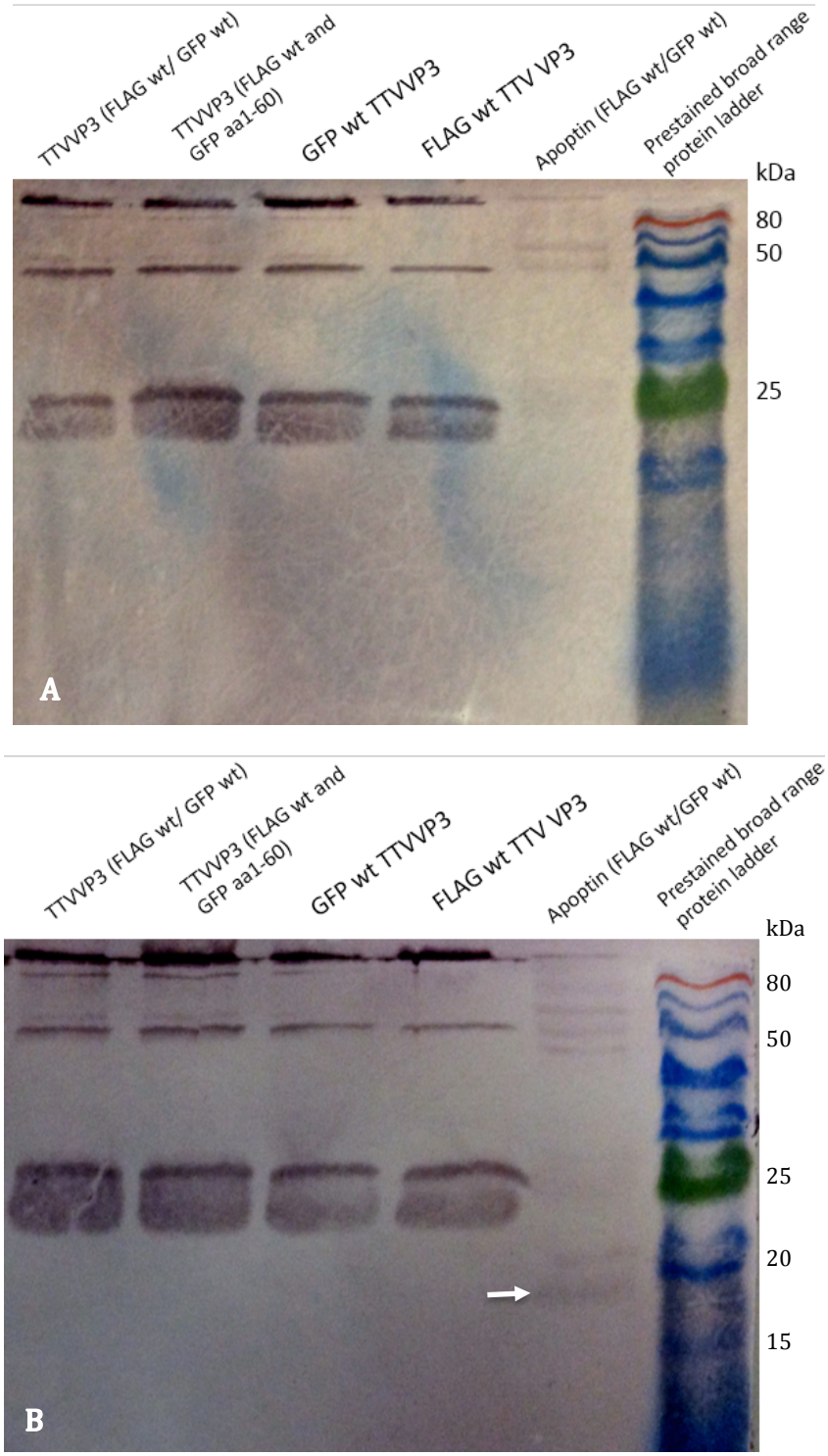

Figure 8: (A) Blotting control and truncated TTV VP3 samples for GFP. Heavy and light chain bands are visible at 55kDa and 25kDa respectively but no other distinct bands are visible except for the Apoptin control. (B) Blotting control and truncated TTV VP3 samples for FLAG. Again, heavy and light chain bands are visible. There is no indication of FLAG around 14kDa, the size of 3X FLAG-fused TTV VP3, while there is a band at 17kDa (white arrow) for the 3X FLAG-Apoptin fusion protein.

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